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Genotoxicity of sub-lethal di-n-butyl phthalate (DBP) in Nile tilapia (*Oreochromis niloticus*)

Aysel Çağlan Karasu Benli[1], Belda Erkmen[2], and Figen Erkoç[3]

Department of Environmental Sciences, Institute of Natural and Applied Sciences, Gazi University, Teknikokullar, Ankara[1], Department of Biology, Faculty of Science and Letters, Aksaray University, Aksaray[2], Department of Biology Education, Gazi University, Teknikokullar, Ankara[3], Turkey

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This study aimed to assess genotoxicity in Nile tilapia (*Oreochromis niloticus*) erythrocytes after exposure to a sub-lethal concentration of 10 mg L^{-1} di-n-butyl phthalate (DBP) for 24 and 96 h. The results showed that mean MN frequencies in both DBP and ethyl methane-sulfonate (EMS, positive control for MN bioassay) groups were significantly different ($p<0.01$) with respect to control and solvent control groups, in both exposure scenarios. When analysing nuclear abnormalities, the frequency of notched nuclei was significantly different ($p<0.05$) but the frequencies of other subtypes did not change. The 96-h exposure led to an increase in the mean frequencies of notched nuclei, and also caused significant differences between MN frequencies in all groups ($p<0.01$). Our findings indicate that sub-lethal DBP concentrations when tested in controlled laboratory conditions have genotoxic potential towards Nile tilapia. Further detailed studies should be done for the determination of the environmental risk assessment for aquatic life since DBP is a high risk contaminant of freshwater and marine ecosystems.

KEY WORDS: *bioassay; genotoxicity; notched nuclei; phthalates*

Overpopulation, lack of efficient sewage-treatment systems, urban construction, industrial and domestic effluents, climate change, port activities, overfishing, and other anthropogenic causes have brought about considerable pollution and habitat destruction problems in coastal areas. These factors have led to contamination with poisonous and genotoxic substances such as pesticides, trace metals, polycyclic aromatic hydrocarbons, polycyclic aliphatic hydrocarbons, plasticizers, and persistent organic pollutants (1), thus severely impacting marine and freshwater ecosystems (2, 3).

Phthalates and their esters (PEs) are widely used in industry as solvents and additives in the polyvinyl chloride production process. However, since the levels of plasticizer recovery and recycling are very low, millions of tons end up in landfills and oceans each year (4). Low-molecular-weight phthalates, such as dibutyl phthalate (DBP; di-n-butyl phthalate, CAS: 84-74-2) and diethyl hexyl phthalate are considered very dangerous substances in the EU REACH regulation. They are classified as category 1B in the Commission Directive 2007/19/EC (cannot be used to make toys, childcare articles, and cosmetics) and risk-reduction measures are required for their safe use. Canada and the United States have also taken regulatory actions restricting their use (5). Furthermore, they pose a particular risk to aquaculture: DBP is used as a solvent in the pesticide formulation of dichlorvos, Aquagard® (6).

The *in vitro* and *in vivo* (tested in several bioindicator species, such as molluscs, plants, amphibians, reptiles, birds, and mammals) micronucleus assays (MN) are relatively simple, validated, reliable, and sensitive tests (7, 8) standardised by the guidelines of international organisations such as the OECD (9). The MN test has been used to evaluate the effects of mutagenic compounds and detect clastogenic (agents which break chromosomes) and aneugenic agents (that induce aneuploidy or abnormal chromosomal segregation) in the cytoplasm of interphase cells in different environmental and ecotoxicological studies (7, 10). An increase in the frequency of micronucleated (MNed) immature erythrocytes is an indication of induced structural or numerical chromosomal and/or the mitotic apparatus aberrations/damage in treated animals. MNi can contain either lagging chromosome fragments or whole chromosomes unable to migrate to the poles during the anaphase stage of cell division. In addition to MN, nuclear abnormalities (NAs) have also been determined in fish cells exposed to genotoxic substances. Cells with two nuclei are known as binucleates. Blebbed nuclei are those with a relatively small evagination of the nuclear membrane, which contains euchromatin. Evaginations larger than those in the blebbed nuclei, which could have several lobes, are classified as lobed nuclei. Nuclei with vacuoles and

Correspondence to: A. Çağlan Karasu Benli, Department of Environmental Sciences, Institute of Natural and Applied Sciences, Gazi University, Teknikokullar, Ankara, Turkey, Tel: +903122023733; Fax: +903122023710, E-mail: *benli@gazi.edu.tr*

appreciable depth into a nucleus that does not contain nuclear material are notched nuclei (11).

Staples et al. (12) have reviewed aquatic toxicity of 18 MNi PEs and reported DBP acute 96-h LC_{50} toxicity values to freshwater fish: toxicity to bluegills was between 0.48 and 2.10 mg L^{-1}; the values for rainbow trout were between 1.2 and 6.47 mg L^{-1}; and between 0.61 and 3.95 mg L^{-1} for fathead minnows. Other freshwater fish tested using DBP were red killifish (48-h LC_{50} of 4.3 mg L^{-1}), zebrafish (96-h LC_{50} of 2.2 mg L^{-1}), and yellow perch (96-h LC_{50} of 0.35 mg L^{-1}). Given the wide ranges of toxicity values of different PEs to aquatic species, it is virtually impossible to develop a generalised guideline for ecotoxicological risk assessment. In general, PEs exert low toxicity to mammals and intermediate toxicity to fish and invertebrates. However, other endpoints and mechanisms such as endocrine disruptor effects are of raising concern (12).

Taken into account the ubiquitous presence of phthalates and their esters, and the possibility of their unwanted release into the environment, there is a continuous need to broaden research focused on the clarification of the mechanisms responsible for their detrimental effects on aquatic organisms. This study intended to fill a gap in the knowledge regarding the sensitivity of Nile tilapia *(Oreochromis niloticus)* towards the putative genotoxic effects of DBP. Nile tilapia was chosen as a test system considering that this fish species (although naturally occurring in Africa) has recently been widely introduced in aquaculture. Furthermore, it is today one of the economically most important farmed freshwater edible epibenthic fish species. Using two exposure scenarios, 24- and 96-h exposure under controlled laboratory conditions, in the present study we evaluated the genotoxic potential of DBP in juvenile Nile tilapia, *Oreochromis niloticus* by employing the MN test, which is nowadays considered one of the most comprehensive and informative genotoxicity endpoints.

MATERIALS AND METHODS

1. Test organism

Juvenile test fish, *Oreochromis niloticus* (Nile tilapia, Perciformes: Cichlidae) were obtained from Çukurova University Freshwater Fish Production Station (Adana, Turkey). For the purpose of the present study we used early life stages of this fish species since they are more sensitive to xenobiotics than adults. A total of 80 specimens were used and their mean weights and lengths were 34.4 ± 4.2 g and 12.9 ± 0.56 cm, respectively.

2. Acclimatisation

Fish were acclimated to laboratory conditions for two weeks before the commencement of the experiments. They were maintained in aerated municipal city water at a population density of 10 fish in 60 L glass aquaria, and fed commercial trout feed (45 % protein) at a daily rate of 2 % of their body weight, at 16/8 h photoperiod. The quality of water was regularly monitored and the values of water temperature, pH, conductivity, dissolved oxygen, and salinity were 23±1°C, 8.98, 576 µS cm^{-1}, 5.7 mg L^{-1}, and 0.30±0.01 ppt respectively. Feeding was stopped 48 h prior to the experiments. The guiding principles for experimental procedures in the Declaration of Helsinki of the World Medical Association regarding animal experimentation were followed in the present study.

3. Exposure conditions

The standardised OECD Testing Guidelines and Turkish National Regulation for Static Bioassays were used. Each exposure model, the 24- and 96-h one, consisted of four tanks: one for the control group (fish were kept in municipal tap water; no further treatment was applied), one for the solvent control group (200 µL acetone), one for the DBP sub-lethal exposure group (10 mg L^{-1} dissolved in acetone, Merck, Germany), and one for the ethyl methanesulfonate (EMS; CAS No. 62-50-0, 5 mg L^{-1}, Sigma, USA) positive control group.

Tanks were constantly aerated during the experiment. The aeration was stopped when dosing of chemicals was performed. The tested chemicals were added into the water of the tanks. The exposure concentration was mainly based on our preliminary range-finding experiments for finding LC_{50} values (reached after 96-h exposure to 50 mg L^{-1} DBP), as was also suggested by other authors in another tilapia species (*Oreochromis mossambicus*) (15). Before the beginning of exposure experiments, the health status of fish was checked on two specimens randomly selected from each aquarium (basic blood parameters as haematocrit, RBC, WBC etc. were checked and necropsy, and subsequent histology examination was routinely performed, but these analyses are not part of the present study). After the health status of fish was confirmed acceptable, we proceeded with the experiments. As mentioned before, the exposures lasted for 24 and 96 h, and during the experiments behavioural changes of fish were also monitored.

4. The MN test

At the end of exposure times, blood samples were collected from the fish under ice anaesthesia by cardiac puncture into heparinised syringes. Thin peripheral blood smears were prepared for the MN assay on the slides previously cleaned with ethyl alcohol and distilled water. After fixation in 96 % ethanol for 20 min, the slides were allowed to air-dry, and then the smears were stained with 5 % Giemsa solution for 20 min. All slides were coded and scored blind. Peripheral blood samples were smeared onto three slides per fish (7) and 1000 cells were scored from each slide (total of 3000 cells per fish) under 1000× magnification. The scoring criteria for MNi and other NAs other than MNi in erythrocytes were done according to

Kirsch-Volders et al. (10) and Cavas and Ergene-Gozukara (11). For a critical review of the methodology cf. Carrasco et al. (16). NAs were of four subtypes: (*i*) binucleated; (*ii*) blebbed nuclei; (*iii*) lobed nuclei; (*iv*) notched nuclei. Nuclear abnormality data were expressed as total NA calculated by the sum of all nuclear abnormalities.

5. Statistical analysis

Statistical significance of the data obtained by the MN test was evaluated using non-parametric statistics (Mann-Whitney U test, and Kruskal Wallis-H test). In all cases, a significance level of 5 % was used.

RESULTS AND DISCUSSION

The present study reports the results of 24- and 96-h acute toxicity of a widely used plasticizer DBP to juvenile Nile tilapia at 10 mg L^{-1} static exposure concentration (*in vivo* experimentation).

No mortality was recorded during the experiments and the fish showed normal behaviour. The weight and total length of the fish did not change with exposure duration ($p>0.05$) nor were there any differences between the EMS, DBP, and control groups in this regard. At the end of 24- and 96-h exposure periods, fish blood samples were collected and MNi and NAs were scored/determined using the MN test. The piscine MN test has been successfully used to evaluate the genotoxic potential of a wide variety of chemical and physical agents; it is even indicated for conditions of chronic exposure *in situ* (7).

The results of MN and NA analyses are given in Table 1 for the 24-h exposure and Table 2 for the 96-h exposure.

In the present study, we observed similar values in control and acetone control groups for both genotoxicity end-points. On the other hand, EMS, used as positive control, caused the highest MN frequency during both exposure times, which confirms the validity of the method used.

24-h exposure

The mean frequencies of MNi observed, both in DBP and positive control (EMS) groups, were high, but the difference between them was not statistically significant (Table 1). However, both mean frequencies of MNi were significantly higher as compared to control and solvent control groups.

After 24-h exposure DBP caused more than twofold increase in NAs compared with the EMS group. Again the differences between these two groups were not significant. However, NA frequencies were significantly different ($p<0.01$) when compared to control group.

We found a statistically significant increase ($p<0.05$) in the frequency of notched nuclei as compared to other subtypes of NA (Figure 1).

96-h exposure

As presented in Table 2, the values of mean frequencies of MNi were comparable to the means obtained after 24-h exposure. The values recorded in DBP and EMS groups significantly differed as compared to both control groups ($p_{DBP}<0.05$) ($p_{EMS}<0.01$). The same was found for NA ($p<0.01$).

Table 1 *Frequency of MNed cells (‰, MN) and nuclear abnormalities (‰, NA) in juvenile Nile tilapia exposed to sub-lethal DBP (10 mg L^{-1}) for 24 h (mean ± S.E.M.; n=10)*

Group	MN (frequency/1000 cells)	Confidence Interval (95 %)	NA (frequency/1000 cells)	Confidence Interval (95 %)
DBP	6.4±1.03*	3.5-9.3	2.8±0.9*	0.1-5.5
Control	1.8±0.5	0.6-3.1	0.5±0.3	-0.38-1.4
Solvent control#	1.2±0.3	0.8-2.6	0.7±0.3	0.2-1.6
Positive control$	10.5±1.7*	6.2-14.8	1.2±0.4	0.1-2.2

A total of 3000 erythrocytes per fish were analysed; S.E.M.=Standard error of the mean;#=acetone; $=EMS
**p<0.01 DBP compared with the control and solvent control groups; positive control with both control groups*

Table 2 *Frequency of MNed cells (‰) and nuclear abnormalities (‰) in juvenile Nile tilapia exposed to sub-lethal DBP (10 mg L^{-1}) for 96 h (mean ± S.E.M., n=10)*

Group	MN (frequency/1000 cells)	Confidence Interval (95 %)	NA (frequency/1000 cells)	Confidence Interval (95 %)
DBP	6.4±1.1*	3.3-9.5	4.0±0.5**	2.5-5.5
Control	2.3±0.7	0.4-4.3	1.0±0.4	0.06-1.9
Solvent control#	1.95±0.5	-1.1-3.4	1.2±0.6	0.1-2.3
Positive control$	9.7±1.6**	5.5-13.9	3.2±1.6	-0.8-7.2

A total of 3000 erythrocytes per fish were analysed; S.E.M.=Standard error of the mean;#=acetone; $=EMS
** p<0.05 DBP compared with the control and acetone control groups*
***p<0.01 DBP compared with the control and acetone control groups; EMS with both control groups*

Similarly as after 24-h exposure, no statistically significant differences were found between mean MNi and NA frequencies observed in DBP and EMS groups.

The 96-h exposure resulted in increased mean frequencies of notched nuclei in DBP- and EMS-exposed group (Figure 1).

We also evaluated whether there were any differences between two exposure times. Since the mean values recorded for the groups of interest were not significantly different, it is concluded that there was no time-effect in DBP toxicity towards Nile tilapia erythrocytes under the experimental conditions studied here.

The piscine MN test using nucleated teleost erythrocytes has recently gained more popularity among researchers since it is also used for engineered/non-engineered nanomaterials, other emerging contaminants such as pharmaceuticals and personal care products, in addition to already known industrial and agricultural pollutants (17-19).

The background erythrocyte micronucleus frequency range for tilapia was previously reported to be 0.3 to 1.9 % (20-22). The values obtained in our control groups are in agreement with this range.

No work has so far been published on the genotoxicity potential of DBP on fish erythrocytes using the MN test in the open literature. DBP as an environmental contaminant and its exposure from food containers is of concern as it represents a risk to reproductive and developmental toxicity (23, 24).

In their study, Abu Zeid and Khalil (25) exposed Nile tilapia fingerlings to DBP at 1.18 mg L^{-1} and 0.59 mg L^{-1} for eight weeks. Using the comet assay, they detected a slightly but not significantly increased level of DNA damage in their gill tissue. Diethyl phthalate (DEP) genotoxicity to another freshwater bioindicator and standard test organism, carp, was evaluated using 0.125, 0.5, 2.0, and 8 mg L^{-1} exposure concentrations for 20 days (26). Although the lowest 0.125 mg L^{-1} exposure dose did not increase MN frequency significantly, all other instances showed significantly higher MN and NA frequencies compared to controls.

The usefulness of Nile tilapia as a model organism in other MN studies was also confirmed previously. For instance, Perera and Pathiratne (22) studied biomarker responses of Nile tilapia exposed to textile industry effluents. They used aged tap water as control and found that frequencies of MNi increased significantly in effluent- and cadmium-exposed groups (22). In another study (20), specimens of Nile tilapia were exposed to contaminated river water and reference/control underground water for 72 h and MN/NA were determined. The results showed a significant increase in genotoxic effects due to insufficient wastewater treatment. Jiraungkoorskul et al. (27) evaluated the sensitivity of three fish species native to Southeast Asia freshwater ecosystems for their use as pollution biomarkers. They used the MN-test and found that Nile tilapia was more sensitive to heavy metal exposure than Butterfish (*Poronotus triacanthus*) and Red-tailed tinfoil barb (*Puntius altus*).

Osman et al. (18) investigated the usefulness of the MN test on Nile tilapia erythrocytes for *in situ* evaluation of the genotoxic potential of the river Nile and found that this fish species was a more suitable bioindicator species than African catfish in the study of genotoxic chemical pollution. The MN test on Nile tilapia erythrocytes was also confirmed suitable for the evaluation of herbicide toxicity (28-31).

Our results demonstrated the suitability of Nile tilapia as a sensitive biomarker of genotoxicity using the micronucleus and nuclear abnormalities test on erythrocytes. Although positive, the results on DBP toxicity call for further investigations on other model systems, and also by employing other sensitive methods. In that regard, comet assay could also be a suitable method. Dobrzyńska et al. (32) performed a comet assay study with male mice and found that DBP was capable of inducing DNA damage in germ cells, but the effect was not statistically significant as compared to controls. However, they also observed other

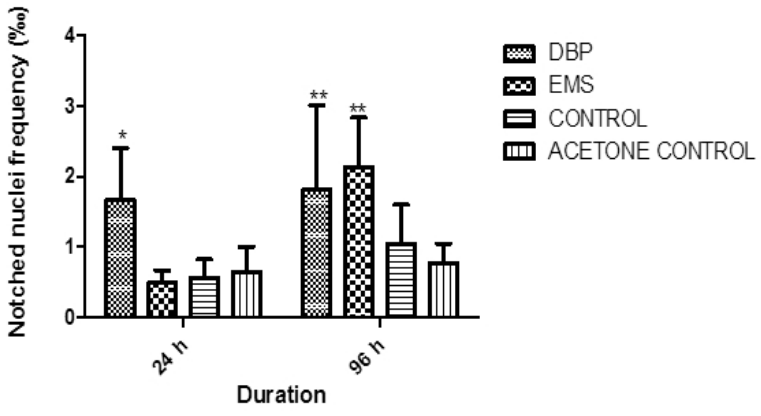

Figure 1 *Frequencies of notched nuclei (mean ± S.E.M) in juvenile Nile tilapia exposed to sub-lethal DBP concentration (10 mg L^{-1}) for 24- and 96-h*
$^{*}p<0.05$ DBP 24 h exposure compared with the control, positive control (EMS) and solvent control (acetone) groups
$^{**}p<0.05$ DBP 96 h exposure and positive control (EMS) compared with the control and solvent control (acetone) groups

effects of DBP, which deserve particular attention, such as drastically reduced sperm motility, changed morphology, and increased formation of MNi in spermatocytes (at the highest dose).

Taking into account wide ranges of toxicity values of different PEs to aquatic species, their low toxicity to mammals and intermediate toxicity to fish and invertebrates it is hard to develop a generalised guideline for ecotoxicological risk assessment. Furthermore, other endpoints and mechanisms such as endocrine disruptor effects are of raising concern. Some species may also be more sensitive to genotoxic effects. Considering that we carried out the study only with Nile tilapia, our results are limited to this species but represent a solid ground for future studies in that regard. Although the exact biological mechanisms of action of phthalates have not been fully elucidated yet, we can conclude that genotoxicity is one of the outcomes of DBP action and it should be taken into consideration when assessing the risks associated with their exposure.

In conclusion, further detailed work on the mechanism of action and genotoxicity potential of DBP and its congeners are necessary for more accurate risk assessment and risk management for this very widely present contaminant.

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Genotoksičnost subletalne koncentracije di-n-butil ftalata (DBP-a) u nilskoj tilapiji (*Oreochromis niloticus*)

Istražena je genotoksičnost subletalne koncentracije 10 mg L^{-1} di-n-butil ftalata (DBP-a) na eritrocitima nilske tilapije (*Oreochromis niloticus*) nakon 24 sata i 96 sati izlaganja riba. Grupne srednje vrijednosti učestalosti mikronukleusa u eritrocitima bile su značajno ($p<0,01$) povišene u skupini izloženoj DBP-u i pozitivnoj kontroli u odnosu na negativnu kontrolu i kontrolu s otapalom u objema vremenskim točkama. Od svih oštećenja jezgrina materijala statistički je značajno odstupala učestalost ($p<0,05$) urezanih jezgri, a učestalost drugih vrsta oštećenja nije se značajno promijenila. 96-satno izlaganje uzrokovalo je porast grupnih srednjih vrijednosti urezanih jezgri te značajne ($p<0,01$) razlike između učestalosti mikronukleusa u svim skupinama. Subletalne koncentracije DBP-a u kontroliranim laboratorijskim uvjetima posjedovale su genotoksični potencijal prema eritrocitima nilske tilapije. Potrebna su daljnja istraživanja kako bi se utvrdio okolišni rizik za vodeni svijet s obzirom na to da je DBP visokorizična onečišćujuća tvar slatkovodnih i morskih ekosustava.

KLJUČNE RIJEČI: *biološki test; genotoksičnost; ftalati; urezana jezgra*